Developments in the prevention of transfusion-associated
graft-versus-host disease

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Summary
The transfusion of blood products can result in a variety of consequences, including transfer of pathogens and the induction of immune responses, such as the almost invariably fatal transfusion-associated graft-versus-host disease (TA-GVHD). Exposure of blood products to γ-irradiation is currently the standard of care for the prevention of TA-GVHD. Regulatory, technical and clinical challenges associated with the use of γ-irradiators are driving efforts to develop alternatives. Initially, pathogen reduction methods were developed to reduce the risk of microbial transmission by blood components. Through modifications of nucleic acids, these technologies interfere with the replication of both pathogens and leucocytes. These methods have been found to be as effective as γ-irradiation in preventing T lymphocyte proliferation and TA-GVHD responses. Additional benefits of pathogen reduction protocols potentially include inhibition of antigen presentation, cytokine production and activation of lymphocytes.

Keywords: blood transfusion, cellular immunology, animal model, graft-versus-host disease.

Transfusion of blood products is used to restore haematopoietic cell populations, such as red blood cells (RBC) and platelets in deficient recipients. However the transfusion of blood products can also result in the transfer of infectious pathogens and leucocytes. The transfused leucocytes can act as antigen-presenting cells and directly or indirectly trigger alloreactive immune responses by the recipient lymphocytes, such as the production of alloantibodies by recipient B cells. Leucocytes, if functional in the transfused blood or blood products, are also capable of generating donor anti-recipient responses with the most severe consequence occurring when cells in the transfused blood product generate unimpeded alloreactive responses to recipient antigens. This response results in transfusion-associated graft-versus-host disease (TA-GVHD), a response that is almost uniformly fatal (Dwyre & Holland, 2008).

TA-GVHD typically presents within 2–30 d following transfusion with an erythematous, maculopapular rash, fever, elevated liver enzymes, often with associated hepatomegaly and jaundice, plus gastrointestinal symptoms, including nausea, vomiting and diarrhoea (Dwyre & Holland, 2008). The initial stages of TA-GVHD are often confused with allergic reactions to drugs or viral infections. The severity of TA-GVHD results from the marked bone marrow aplasia caused by the donor anti-recipient response. The resulting neutropenia renders the patient highly susceptible to infection. The bone marrow aplasia presents a difficult hurdle to overcome when attempting to treat the patient with ongoing TA-GVHD. For these reasons, emphasis has been placed on preventing the development of TA-GVHD.

Inhibition of alloreactive T cell proliferation has been the focus of TA-GVHD prevention as these cells are considered the primary effector population present in the blood product responsible for TA-GVHD (Luban, 2001). While it is possible that alloantibodies produced by donor B cells could generate TA-GVHD, there are only limited reports of the occurrence of this phenomenon (Gilstad et al, 2012). The basis for alloreactivity is the cross-reactivity of the T cell receptor that is facilitated by its flexibility when binding to allogeneic human leucocyte antigen (HLA) molecules expressing particular peptides (Smith et al, 2012). This property results in a large percentage (1–10%) of T cells responding directly to any given allogeneic major histocompatibility complex (MHC) antigen and potentially generating a response of large magnitude.

Because transfused blood products are not usually typed for HLA antigens, a transfusion could potentially result in both recipient anti-donor as well as donor anti-recipient alloreactive responses. The outcome from these competing responses depends on a number of factors. These factors include the number of donor leucocytes present in the blood product, the HLA antigens expressed by both donor and recipient cells, and the immune competence of the recipient and donor lymphocytes. All factors being equal, usually there are significantly more recipient leucocytes than donor leucocytes, resulting in elimination of the donor lymphocytes. Assessment of donor leucocyte survival in transfusion recipients of allogeneic blood units found that 99.9% of the donor...
leucocytes were eliminated within 3 d after transfusion, with the remaining cells eliminated within a week (Lee et al., 1995). Rapid elimination was also found when large numbers of murine spleen cells were injected into naive allogeneic recipients (Fast et al., 1995). In this model, allogeneic donor spleen cells were eliminated in 3 d by recipient CD8+ cells (Fast, 1996). Donor CD4+ cells were found to be important stimulators of the recipient CD8+ responses (Fast, 2000). In the absence of recipient CD8+ cells, the production of anti-donor alloantibodies was important for the elimination of donor cells (Fast, 2000). Based on these observations in murine recipients, prolonged persistence of the donor lymphocytes and donor anti-recipient alloreactive responses would be expected in situations in which the recipient has suppressed or absent CD8+ and B cell responses. This could occur because of inherited genetic deficiencies or by inhibition of the production or function of these cells by immunosuppressive drugs. Another possible scenario for decreased function of the recipient immune system is the immunosenescence associated with aging (Lang et al., 2012). It would be expected that individuals with these deficiencies would be more susceptible to the development of TA-GVHD. In contrast, the continued and prolonged presence of functional CD8+ cells in human immunodeficiency virus (HIV)-infected individuals capable of eliminating donor leucocytes may provide an explanation for the paucity in the number of TA-GVHD cases reported in these individuals (Dwyre & Holland, 2008) and is consistent with the lack of prolonged survival of transfused white blood cells (WBC) observed in these individuals (Kruskall et al., 2001).

Diminished recipient anti-donor responses are also seen when the antigenic disparity between donor and recipient is such that donor alloreactive cells recognize recipient HLA antigens as foreign but the donor alloantigens are seen as self by recipient lymphocytes. This would occur, for example, when the donor is homozygous for one of the HLA haplotypes expressed by the recipient. While this donor/recipient combination would occur infrequently at random, it is more likely when relatives are donating the blood or in more homogenous populations. For example, the Japanese population is a relatively homogeneous population and this donor/recipient combination has been calculated to occur at 1 in 874 unrelated blood transfusions (Ohto et al., 1992). In Caucasian populations this frequency is ninefold less (Ohto et al., 1992; Ohto & Anderson, 1996). As expected, an increased risk of TA-GVHD has been observed in the Japanese population. While the frequency of TA-GVHD is unknown, partly because of misdiagnosis and underreporting, it is clear that the frequency of TA-GVHD does not approach the calculated incidence of 1 in 7174 in Caucasians. Recent studies have documented that GVHD or graft rejection can occur in HLA-matched transplants (Sabloff et al., 2011). Presumably these responses could be triggered by disparities in minor histocompatibility antigens (Goulmy, 2006; Spellman et al., 2009). One possible reason for the lower than expected frequency of TA-GVHD in this genetic donor/recipient combination is that recipient responses to donor cells expressing identical MHC molecules but differing minor histocompatibility antigens could result in elimination of these donor cells, preventing donor anti-recipient responses.

Prevention of TA-GVHD using leucoreduction or γ-irradiation

Because of the difficulty in reversing TA-GVHD responses once they have been initiated, focus was placed on identifying approaches capable of preventing the onset of TA-GVHD. Initial studies targeted the depletion or inhibition of the T cells present in blood products prior to the transfusion. Leucoreduction has been found to reduce the number of leucocytes present in the blood products by 5 logs or more. The reduced numbers of WBC in these units has reduced the incidence of TA-GVHD (Hayashi et al., 1993; Stainsby et al., 2006), however there still have been reports of TA-GVHD developing in recipients who received leucoreduced blood products containing very limited numbers of leucocytes (Akahoshi et al., 1992). The other approach that was taken was to identify methods that would inhibit the proliferation of T lymphocytes without affecting the other cells in the blood product. Investigators initially focused on the use of γ-irradiation. In vitro assays measuring proliferative responses in a mixed lymphocyte culture (MLC) and/or quantifying the number of cells responding in a limiting dilution assay (LDA) were used to determine the doses of γ-irradiation needed to effectively prevent lymphocyte proliferation (Pelszynski et al., 1994; Luban et al., 2000). In the LDA, varying numbers of responding cells are placed in wells containing feeder cells with a stimulatory medium. The wells containing T cell clones are counted after a period of time, usually several weeks. The frequency of responding cells is determined by Poisson statistics. Results of these in vitro studies led to the adoption of a dose of 25–30 Gy γ-irradiation as a standard for the inactivation of T lymphocytes in blood products.

Because of the high frequency of TA-GVHD in Japanese populations, uniform γ-irradiation of blood products was implemented in Japan in 2000 and there has been no documented cases of TA-GVHD since that time (Otsubo & Yamaguchi, 2008). In the United States of America there is selective γ-irradiation of blood products and, because each institution needs to set their own guidelines, there is ongoing discussion about which patients should receive γ-irradiated blood products. American Association of Blood Banks (AABB) standards require that (i) any patient at risk for TA-GVHD, (ii) patients receiving blood donated by a blood relative and (iii) patients receiving blood products selected on the basis of HLA compatibility should receive irradiated products (King & Ness, 2011). Patients at risk for TA-GVHD include fetuses receiving intrauterine transfusion, premature infants, neonates who require RBC exchange, patients with
haematopoietic malignancies and certain solid tumours, patients receiving intensive chemotherapy or purine antimetabolites, patients receiving progenitor cell transplantation, granulocyte recipients and patients with congenital immunodeficiency syndrome (King & Ness, 2011).

While the standard of care for preventing TA-GVHD has been to expose blood products to γ-irradiation, several drawbacks to this method have become apparent over time. Irradiated RBC have a decreased shelf-life because of increased membrane permeability leading to haemolysis and potassium leakage. Another drawback is that while the use of γ-irradiation is effective at inhibiting TA-GVHD, it is much less effective in preventing other types of immune responses, such as the production of alloantibodies (Ohto, 1997). Another drawback noted recently is that the caesium source irradiators installed in blood banking facilities are now considered a liability because of concern over the potential use of caesium for bioterrorist activities (Mintz, 2011). As a result of this concern, regulation of the individuals operating the irradiator has increased significantly as well as strictly mandated protocols for using the irradiator. This has led to a search for alternative methods for preventing TA-GVHD, with possibilities including the use of X-irradiation as well as pathogen reduction protocols.

**Prevention of TA-GVHD using pathogen reduction protocols**

Concern for the transmission of unknown pathogens by transfused blood products led to the development of several pathogen reduction protocols. The general principle of these protocols is the modification of nucleic acids in ways that prevent replication. In addition to determining the effectiveness of the treatments in reducing the levels of pathogens, concerns associated with the development of these protocols include the effects on the viability of the blood cells and the possibility that these protocols will introduce new antigenic epitopes that will result in recipient immune responses causing the patient to become refractory to the treated cells. A summary of the different pathogen reduction protocols is shown in Table I.

While these pathogen reduction protocols could clearly impact the replication of pathogens it was also observed that they could interfere with the replication of leucocytes. This has led to exploration of the use of these pathogen reduction protocols as alternatives to γ-irradiation in preventing TA-GVHD. As early studies testing these protocols were being conducted, it became clear that the ability to prevent proliferation of T lymphocytes in the MLC and LDA assays, the same assays used to define the dose of γ-irradiation, was not going to be sufficient to convince the transfusion medicine community to consider adopting them. In part this may have been based on the desire to maintain the status quo based on the capital investment in irradiators. As a result, studies exploring the ability of pathogen reduction protocols to prevent proliferation of T lymphocytes also led to an exploration of different in vivo models of TA-GVHD in order to compensate for the inability to conduct clinical trials because of ethical constraints.

Initially the parent into F1 murine GVHD model was studied. This donor/recipient combination is similar to the donor/recipient combination often associated with the development of TA-GVHD (MHC homozygous donor/MHC heterozygous recipient). The parental donor strain is homozygous for H-2 while the F1 recipient shares one haplotype with the donor while the other H-2 haplotype is allogeic. In this model, if one injects a relatively large number of parental splenocytes, it is possible to induce GVHD in naive recipients even if the recipient has not been treated in any way (Puliaev et al, 2005). In the most often studied combination it has been found that injection of C57BL/6 splenocytes into B6D2F1 recipients results in an acute form of GVHD characterized by donor anti-recipient CTL and by marrow aplasia. In contrast, the use of splenocytes from the other parent, DBA/2, results in a chronic form of GVHD that resembles lupus and is characterized by hyperproliferative response and high levels of autoantibodies (Via, 2010). Parental splenocytes capable of inducing acute GVHD were treated with INACTINE (PEN 110), an ethyleneimine derivative able to bind to nucleic acids, or were treated with amotosalen, a modified psoralen, in the presence of ultraviolet (UV) light and the GVHD response was measured following

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**Table I. Pathogen reduction protocols.**

<table>
<thead>
<tr>
<th>Pathogen reduction agent</th>
<th>Company</th>
<th>Products treated</th>
<th>Current use</th>
</tr>
</thead>
<tbody>
<tr>
<td>INACTINE (PEN110)</td>
<td>V. I. Technologies (Watertown, MA, USA)</td>
<td>Red blood cells</td>
<td>Halted</td>
</tr>
<tr>
<td>Intercept (S-59+ ultraviolet-light)</td>
<td>Cerus (Concord, CA, USA)</td>
<td>Platelets</td>
<td>Used in Europe</td>
</tr>
<tr>
<td>S-303</td>
<td>Cerus</td>
<td>Plasma</td>
<td></td>
</tr>
<tr>
<td>Mirasol (Riboflavin + ultraviolet-light)</td>
<td>TerumoBCT (Lakewood, CO, USA)</td>
<td>Red blood cells</td>
<td>In development</td>
</tr>
<tr>
<td>Methylene blue + visible light</td>
<td>MacoPharma (Mouvaux, France)</td>
<td>Platelets</td>
<td>Used in Europe</td>
</tr>
<tr>
<td>Solvent detergent</td>
<td>Octapharma (Vienna, Austria)</td>
<td>Plasma</td>
<td>Used in Europe</td>
</tr>
</tbody>
</table>

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British Journal of Haematology, 2012, 158, 563–568
injection of the treated parental splenocytes into F₁ recipients (Grass et al., 1999; Fast et al., 2002). These studies indicated that treatment of donor splenocytes with these two different pathogen reduction protocols were able to inhibit GVHD responses in this murine model. Reservations about using the P into F₁ GVHD model primarily focused on the fact that this model tested the in vivo responses of murine lymphocytes and not human lymphocytes.

To measure the in vivo responses of human lymphocytes instead of murine lymphocytes, the ability of human lymphocytes to induce xenogeneic GVHD responses when injected into immunodeficient mice was tested. Initial studies examined the xenogeneic GVHD responses in severe combined immunodeficiency (SCID) mice (lacking B and T cells) reconstituted with human peripheral blood mononuclear cells (PBMC). While chimaerism was not observed in a large fraction of the recipient SCID mice, GVHD did develop in mice that exhibited significant engraftment of human T cells (Hoffmann-Fezer et al., 1993). Subsequent experiments found that increasing the donor cell dose or administration of interleukin (IL)-15 to the SCID mice injected with human PBMC potentiated the xenogeneic GVHD responses (Sandhu et al., 1995; Roychowdhury et al., 2005). Because of the sporadic induction of xenogeneic GVHD when PBMC were injected into SCID mice, investigators moved to the use of Rag2<sup>−/−</sup>γC<sup>−/−</sup> double knockout mice that had also received a 350 cGy irradiation dose as recipients (van Rijn et al., 2003). These recipient mice were more immunocompromised than SCID mice because they lacked B, T and natural killer (NK) cells and the 350 cGy irradiation reduced myeloid cell function. As a result, there was improved engraftment of human lymphocytes with a corresponding increase in the incidence of xenogeneic GVHD. A series of experiments tested the ability of human PBMC treated with PEN110 or riboflavin + UV light to induce xenogeneic GVHD in these recipient mice (Fast et al., 2004, 2006), the results of which demonstrated that these treatments did prevent the development of xenogeneic GVHD. Further analysis of the mice receiving untreated cells indicated that the mice undergoing xenogeneic GVHD exhibited high plasma levels of γ-interferon (IFN-γ) and moderate levels of IL-10 when euthanized. There were also high levels of human IgG and IgM present in the plasma that were able to bind to mouse RBC and platelets. The reconstituting human lymphocytes were shown to be primarily T lymphocytes.

A more recent paper examined the xenogeneic GVHD responses of human PBMC injected into non-obese diabetic (NOD)-scid IL2r<sup>−/−</sup> recipient mice (King et al., 2009). Like the Rag2<sup>−/−</sup>γC<sup>−/−</sup> double knockout mice, the NOD-scid IL2r<sup>−/−</sup> mice lack B, T and NK lymphocytes. In addition, the NOD background also provides a C5 deficiency in the complement cascade along with impaired myeloid function. The results of these studies showed that as few as 5 × 10⁶ donor PBMC were able to reproducibly generate xenogeneic GVHD in these recipients. The rate of xenogeneic GVHD development was accelerated by giving the recipient mice 200 cGy of irradiation prior to the injection of PBMC.

In a recently published series of experiments, treatment of fresh whole blood was performed within 6 h of collection using riboflavin + UV light, γ-irradiation (25 Gy) or left untreated. The PBMC isolated from each of the aliquots were tested for their ability to proliferate in response to the T cell mitogen, phytohaemagglutinin, or to bound anti-CD3 as well as quantification of proliferation using the LDA assay. It was found that treatment with riboflavin + UV light was as effective as γ-irradiation in inhibiting T cell proliferation (Fast et al., 2012). In a second series of experiments, PBMC isolated from treated or untreated fresh whole blood (six different donors) were injected into the NOD-scid IL2r<sup>−/−</sup> mice (10 mice per group, 10–20 × 10⁶ PBMC) and the development of xenogeneic GVHD was monitored by observing and weighing the mice at least 3 times a week. The mice were euthanized when they exhibited >15% weight loss or developed a hunched appearance with ruffled fur. The results indicated that the recipients of untreated PBMC began developing xenogeneic GVHD by day 9–12 and that 55/60 recipients developed xenogeneic GVHD by the time that the experiments were terminated (Fast et al., 2012). None of the mice injected with PBMC treated with riboflavin + UV light or γ-irradiation developed xenogeneic GVHD or demonstrated any evidence of reconstitution with human cells. Characterization of the plasma from the recipient mice injected with untreated PBMC found that there were high levels of IFN-γ and moderate levels of IL-10 and, occasionally, IL-5. High levels of human immunoglobulin (Ig)G and IgM were also detected. These results indicated that xenogeneic GVHD could be induced more rapidly and uniformly with fewer PBMC in NOD-scid IL2r<sup>−/−</sup> recipients, identifying this model as the best to date for measuring the effects of various treatments on in vivo human T cell responses, responses that mimic those seen in TA-GVHD.

The cumulative data from these studies indicate that any of the pathogen reduction protocols were able to inhibit in vitro T cell proliferation as effectively as γ-irradiation using MLC and LDA assays. Consistent with the in vitro results, almost all of the protocols were tested and found to inhibit GVHD in the P into F₁ murine model or in xenogeneic GVHD models. These results indicate that pathogen reduction protocols should be able to substitute for γ-irradiation in preventing TA-GVHD. However, there are additional parameters that also need to be evaluated, which include: (i) the quality of the treated blood cells (ii) whether the treatments result in new antigenic epitopes that trigger immune responses (iii) Additional benefits that can be gained from treatment with the pathogen reduction protocols.

The use of PEN110-treated RBC was stopped when use of these products induced antibodies to treated RBC in sickle cell patients receiving chronic transfusions or in a subset of patients who received transfusions acutely as a result of sur-
gery (Solheim, 2008). The production of these antibodies was associated with inflammation. Sickle cell patients have a chronically inflamed immune system (Krishnan et al, 2010). Antibodies were also observed in patients who developed an infection following transfusion of the treated blood products. The appearance of new epitopes after PEN110 treatment appeared to be the consequence of membrane perturbations exposing additional epitopes.

Treatment of platelets and plasma with amotosalen plus UV light did not result in the production of neoantigens (Lin et al, 2005) and this protocol is currently used clinically in Europe and a number of other sites. A different compound, S-303, was used for the treatment of RBC and a subset of patients (both control and test) were found to express antibodies before or after transfusion that bound to S-303 treated RBC (Benjamim et al, 2005). These findings resulted in the development of a modified protocol now being tested clinically.

The treatment of blood products with riboflavin + UV light has been found to inhibit cytokine production, activation and antigen presentation of the WBC as well as proliferation, which indicates that the use of this protocol could provide additional benefits (Marschner et al, 2010). The ability to effectively treat fresh whole blood with this protocol could theoretically permit the use of fresh whole blood directly for transfusion or fractionation of the fresh whole blood into its different components following a single treatment. Treatment of platelets using this protocol is presently ongoing in Europe and no induction of antibodies has been reported to date.

The results of the in vitro studies and in vivo models indicate that pathogen reduction protocols are as effective as γ-irradiation in preventing TA-GVHD. While ethical constraints make it impossible to conduct clinical trials, vigilance studies to determine the consequences, including the onset of TA-GVHD, of receiving blood products treated with pathogen reduction protocols are ongoing and to date no documented cases of TA-GVHD have been reported. Future studies and these ongoing studies will help determine the effectiveness of these treatments in the prevention of TA-GVHD.

Increasing regulatory, technical and clinical challenges associated with the use of γ-irradiators are driving efforts to develop alternatives (Mintz, 2011). The experimental results indicate that pathogen reduction protocols are as effective as γ-irradiation in preventing T cell proliferation using both in vitro and in vivo assays. The ability to substitute the use of these protocols for γ-irradiation will depend on the quality of the treated blood products and their neoantigenicity following treatment, but the treatments may also provide additional benefits. This enhanced inhibition could potentially provide additional benefits by preventing immune consequences, such as the induction of alloantibodies and cytokine-mediated effects.

Acknowledgements

Loren D. Fast has received research grant funding from V. I. Technologies and from TerumoBCT (previously known as Navigant and then CaridianBCT).

Author contributions

Loren D. Fast wrote the paper.

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Review


